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Binding sites of ZNC(C)PR, a pentapeptide fragment of argipressin, in rat brain

WU Jian-Hua, DU Yu-Cang[1] (*Shanghai Institute of Materia Medica; [1]Shanghai Institute of Biochemistry, Chinese Academy of Sciences, Shanghai 200031, China*)

AIM: To study the binding sites of ZNC (C) PR, a C-terminal pentapeptide fragment of argipressin, in rat brain. **METHODS:** Using radioligand assay and radioligand [^{35}S]ZNC (C) PR. **RESULTS:** It was found that there exist native binding sites bound ZNC(C)PR with high affinity in a saturable, reversible and specific manner. Scatchard and kinetic analyses showed that these sites were homogeneous with a K_d value of 1.69 ± 0.16 nmol·L^{-1} (in the presence of $MgCl_2$ 10 mmol·L^{-1}). The binding of ZNC(C)PR to the sites was at a higher level in the brain regions (such as amygdala, cortex,) and was affected by Mg^{2+}. **CONCLUSION:** These binding sites represented a new type of receptors and mediated the action of ZNC(C)PR on memory.

KEY WORDS ZNC(C)PR; neuropeptides; neuropeptide receptors; brain; radioligand assay

ZNC (C) PR, a natural C-terminal pentapeptide of rat brain produced enzymatically from argipressin (Arg)[1], has a great potency of promoting rat learning and memory, without pressor and antidiuretic activities[2-4]. ZNC(C)PR is a new neuropeptide with possibly a special action on learning and memory, but we did not find any report about its specific binding sites in brain. In this paper, we studied the specific binding sites of ZNC(C)PR in rat brain.

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Pyroglutamyl-Asn-Cys-Pro-Arg-COOH
|
H-Cys-COOH

ZNC(C)PR

MATERIALS AND METHODS

All chemicals were of AR grade, and the peptides were synthsized in this laboratory by a stepwise solid-phase method, purified by repeated chromatography on a Sephadex G15 column and reverse-phase HPLC as described previously[4,5].

Preparation of radioactive ligand ZNCPR, the precursor of ZNC(C)PR, was oxidized with iodine (dissolved in 0.25 % acetic acid) in the presence of [^{35}S]cysteine (Amersham Co, 37 PBq·mol^{-1}) to form an asymmetrical disulfide. The products were purified on reverse-phase HPLC. The elute was monitored by uv absorbance at 210 nm, and 1.0 mL (1.0 min) fractions were collected. A 10-μL sample of each fraction was subjected to liquid scintillation counting. The fractions containing [^{35}S]ZNC(C)PR were pooled and stored at −80 C.

Tissue preparation Wistar rats, ♂ weighing 180±s 20 g, were killed by cervical dislocation. Brain regions were isolated[6] and homogenized by 6 strokes of a glass homogenizer with a teflon pestle in 20 mL of ice-cold buffer A (pH 7.4, containing sucrose 0.25 mol·L^{-1}, HEPES 1.0, $MgCl_2$ 2.0, PMSF 0.1 mmol·L^{-1}). The homogenate was centrifuged at 1 000×g for 10 min. The pellet was resuspended in buffer A, and centrifuged again at 15 000×g for 25 min, and the final pellet was resuspended in ice-cold buffer B (pH 7.4, containing HEPES 20, NaCl 235, KCl 10, $MgCl_2$ 10 mmol·L^{-1}). All procedures were carried out under 0—4 C. Protein concentration was determined colorimetrically[7] using bovine serum albumin as standard.

Binding assays Binding assays were performed in 7 mL polypropylene centrifuge tubes pretreated with dimethyl dichlorosilane. Membrane aliquots con-

taining 0.25 — 1.0 mg of protein were incubated in 0.25 mL HEPES 20 mmol·L^{-1} buffer (pH 7.4) containing NaCl 235, KCl 10, $MgCl_2$ 10 mmol·L^{-1}, 0.1 % BSA, enzyme inhibitors (leupeptin 10, aprotinin 20 mg·L^{-1}, amstatin 20 μmol·L^{-1}), and varying amounts of radioactive ligand. Control tubes also contained ZNC(C)PR 10 μmol·L^{-1} to determine nonspecific binding.

The incubation was performed at 25 C for 20 min, and the reaction was stopped by adding 3 mL of ice-cold buffer C (pH 7.4, containing HEPES 20, NaCl 235, KCl 10, $MgCl_2$ 10 mmol·L^{-1}, 0.1 % BSA).

Bound labeled ZNC(C)PR was separated by filtration through Whatman glass microfiber filters (GF/C). Filters were rinsed by 4 mL of buffer C thrice, and dried at 50 ℃. Radioactivity retained on the filters was counted by a liquid scitillation counter.

All experiments were performed under duplicate tubes 3 times. The data were expressed as $\bar{x} \pm s$.

RESULTS

Saturation analysis At pH 7.4, in the presence of $MgCl_2$ 10 mmol·L^{-1}, ZNC(C)PR was bound in a saturable manner to hippocampal synaptic plasma membranes ($n = 3$) (Fig 1). The binding approched saturation at [^{35}S] ZNC(C)PR 8 nmol·L^{-1}. Scatchard analysis of the data revealed that the binding sites had a K_d of 1.69±0.16 nmol·L^{-1} and a B_{max} of 19.0± 1.2 fmol/mg protein (Fig 2). The Scatchard plot showed a linear line (r = 0.998).

Kinetic analysis of association and dissociation The time courses of ZNC(C)PR association with and dissociation from the hippocampal synaptic plasma membranes were observed (Fig 3).

In the first 5 min after mixing the radioactive ligand with a membrane preparation, the association was rather quick at first, gradually slowed down, and reached an equilibrium at 15 min. After adding the unlabled ZNC(C)PR 10 μmol·L^{-1} into the reaction

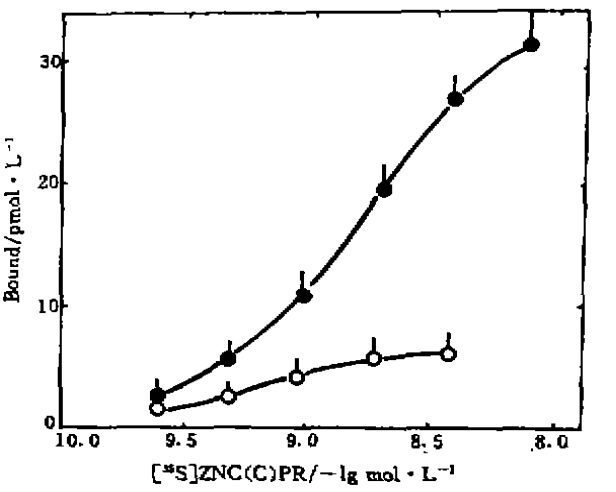

Fig 1. Binding of [^{35}S]ZNC(C)PR to hippocampal synaptic plasma membranes (0.64 mg protein) of rats in the absence (○) or presence (●) of Mg^{2+} 10 mmol·L^{-1}. Nonspecific binding was determined by addition of unlabeled ZNC(C)PR 10 μmol·L^{-1}. $n = 3$, $\bar{x} \pm s$.

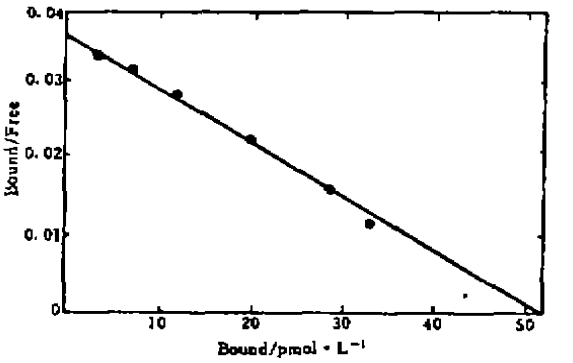

Fig 2. Scatchard analysis of binding of [^{35}S]ZNC(C)PR to hippocampal synaptic plasma membranes of rats.

tube, the dissociation was rapid in the first 10 min, followed by a slow dissociation phase (n = 3). The kinetic data were analyzed on a semilogarithmic plot. Both the association and dissociation courses were linear, indicating that these time courses conform to the first order kinetics. The association rate constant (K_1) and dissociation rate constant (K_2) were

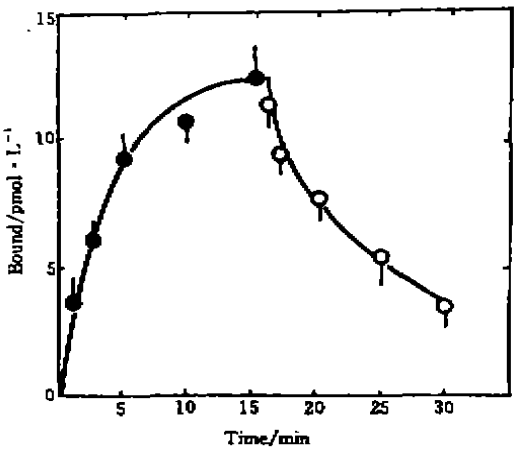

Fig 3. [^{35}S]ZNC(C)PR (2 nmol · L^{-1}) association (●) with and dissociation (○) from hippocampal synaptic plasma membranes (0.25 mg protein). $n=3$, $\bar{x}\pm s$.

calculated as 58 ± 3 pmol · L^{-1} · min^{-1} and 0.060±0.002 min^{-1}, respectively. The equilibrium dissociaton constant (K_d) was deduced as 1.03±0.06 nmol·L^{-1} from the K_1 and K_2.

Competition analysis The inhibition of [^{35}S]ZNC(C)PR 2 nmol ·L^{-1} binding to hippocampal synaptic plasma membrane was investigated by several unlabeled analogs (Fig 4).

The unlabeled ZNC (C) PR was most effective in competitive inhibition of [^{35}S]ZNC(C)PR binding to hippocampal plasma membranes, with a K_i value of 3.7±0.87 nmol · L^{-1}. DDAVP, a Arg analog having memory promoting and antidiuretic activities without effect on BP, was also effective with a K_i value of 363 ± 87 nmol · L^{-1}, but weaker than that of ZNC (C) PR. While Arg, [ZNCPR]$_2$, and ZDC(C)PR were not competitive with the binding of [^{35}S]ZNC(C)PR even at a concentration of 10 μmol·L^{-1}.

Effect of $MgCl_2$ on binding In control experiment without Mg^{2+} in the incubation mixture, the binding capacity was 1.6±0.5

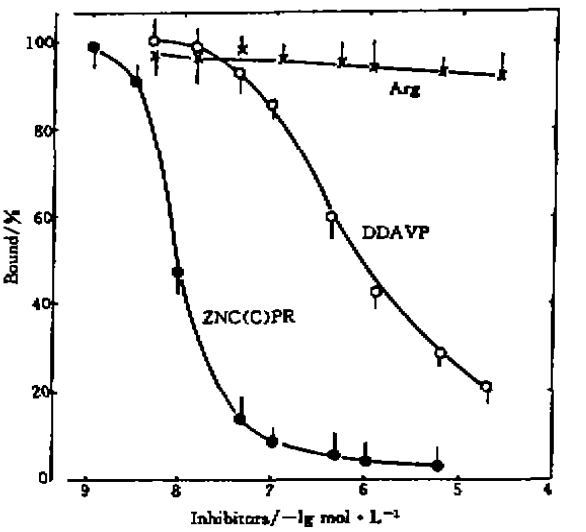

Fig 4. Inhibition of [^{35}S]ZNC(C)PR (2 nmol·L^{-1}) binding to hippocampal synaptic plasma membranes by unlabelled peptices. $n=3$, $\bar{x}\pm s$.

fmol/mg protein. In experiment with $MgCl_2$ 10 mmol·L^{-1} in the incubation mixture, the binding capacity reached 19.0±1.2 fmol/mg protein, which was 10 times higher than the control ($n=3$) (Fig 1).

Regional dissection Using [^{35}S]ZNC(C)PR 8 nmol·L^{-1} to determine the distribution of [^{35}S]ZNC(C)PR binding sites in brain, the binding sites were widely distributed in the rat brain, more abundant in the amygdala, hypothalamus, and cortex; moderate in the septum and hyppocampus; and almost absent in the cerebellum (Tab 1).

DISCUSSION

ZNC(C)PR was a new neuropeptide with a greater potency in promoting rat memory than that of Arg and DDAVP, and without effect on the BP and urine formation[2,3,4]. The present study provide evidences for the existence of native specific binding sites of ZNC (C)PR. These sites are widely distributed in rat brain, but more abundant in the brain re-

Tab 1. Concentrations of [^{35}S]ZNC(C)PR binding sites in brain. $n=3$, $\bar{x}\pm s$.

Brain region	Concentration (fmol/mg protein)
Amygdala	29.3±0.8
Hypothalamus and hypophysis	29.0±0.9
Cortex	24.1±0.5
Nucleus caudatus	21.6±1.0
Nuclei tractus solitarii	21.3±1.2
Septum	19.6±0.7
Superior & inferior colliculi	17.7±1.1
Hippocampus	15.7±0.6
Medulla oblongata	10.2±0.7
Cerebellum	5.5±0.9

gions related to memory and learning. The binding of ZNC(C)PR to these sites has characteristics of saturability, reversibility and specificity, and is affected by Mg^{2+}. As is well known, these features are essential for identifying a receptor. On the basis of the above mentioned facts, therefore, it was suggested these binding sites was receptors. In addition, Scatchard and kinetic analyses of the binding of ZNC(C)PR showed that the sites are uniform. In competition assay, ZNC(C)PR is most effective in inhibiting [^{35}S] ZNC(C)PR binding, DDAVP is less potent, while [ZNCPR]$_2$ and ZDC(C)PR, both of which having no action on memory, fail to displace the [^{35}S] ZNC(C)PR. These results were consistent with their biological activities in the memory assay[2-4]. But it was interesting that Arg *per se* was also unable to inhibit the binding of [^{35}S]ZNC(C)PR. Therefore, the specific binding sites of ZNC(C)PR was native receptors of its own, different from those of Arg ever found. It was proposed that ZNC(C)PR exerted a selective action on memory via the receptors of its own, and Arg influenced memory, at least partly, by its fragment ZNC(C)PR naturally produced enzymatically in the brain.

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精氨酸加压素 C-端五肽片段 ZNC(C)PR 在大鼠脑内的结合位点

吴建华，杜雨苍[1]（中国科学院上海药物研究所，[1]上海生物化学研究所，上海200031，中国）

目的：研究精氨酸加压素 C-端五肽片段 ZNC(C)PR 在大鼠脑内的结合位点。**方法**：用 [^{35}S]ZNC(C)PR 和放射配位体测定法；**结果**：发现大鼠脑内存在 ZNC(C)PR 的独特结合位点，以饱和性、可逆性、特异性和高亲和方式与 ZNC(C)PR 结合，其 K_d 值为1.69±0.16 nmol·L^{-1}，此结合位点在脑内分布较广，以杏仁区和大脑皮层等部位含量较高，而在小脑很低。**结论**：这一结合位点可能代表一新受体，并介导 ZNC(C)PR 的记忆促进作用。

关键词 ZNC(C)PR；神经肽；神经肽受体；脑；放射配位体测定